

Human Thyroid-stimulating Hormone (hTSH) Subunit Gene Fusion Produces hTSH with Increased Stability and Serum Half-life and Compensates for Mutagenesis-induced Defects in Subunit Association*

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The human thyroid-stimulating hormone (hTSH) subunits α and β are transcribed from different genes and associate noncovalently to form the bioactive hTSH heterodimer. Dimerization is rate-limiting for hTSH secretion, and dissociation leads to hormone inactivation. Previous studies on human chorionic gonadotropin (hCG) and human follicle-stimulating hormone had shown that it was possible by subunit gene fusion to produce a bioactive, single chain hormone. However, neither the stability nor the clearance from the circulation of such fused glycoprotein hormones has been studied. We show here that genetic fusion of the hTSH α - and β -subunits using the carboxyl-terminal peptide of the hCG β -subunit as a linker created unimolecular hTSH whose receptor binding and bioactivity were comparable to native hTSH. Interestingly, the fused hTSH had higher thermostability and a longer plasma half-life than either native or dimeric hTSH containing the hCG β -subunit-carboxyl-terminal peptide, suggesting that dimer dissociation may contribute to glycoprotein hormone inactivation *in vivo*. In addition, we show for the first time that synthesis of hTSH as a single polypeptide chain could overcome certain mutagenesis-induced defects in hTSH secretion, therefore enabling functional studies of such mutants. Thus, in addition to prolongation of plasma half-life, genetic fusion of hTSH subunits should be particularly relevant for the engineering of novel analogs where desirable features are offset by decreased dimer formation or stability. Such methods provide a general approach to expand the spectrum of novel recombinant glycoprotein hormones available for *in vitro* and *in vivo* study.

Thyroid-stimulating hormone (TSH)¹ belongs to the glycoprotein hormone family, which also includes the gonadotropins

follicle-stimulating hormone (FSH), luteinizing hormone, and chorionic gonadotropin (CG). These hormones exist as heterodimers composed of a common α -subunit, which is noncovalently linked to a hormone-specific β -subunit (1). Crystallization of hCG had revealed that both subunits have a similar overall structure with a central cystine knot motif (2, 3). Therefore, the glycoprotein hormones are now considered members of the cystine knot growth factor superfamily that includes a variety of structurally related dimeric growth factors, such as nerve growth factor, platelet-derived growth factor, vascular endothelial growth factor, and transforming growth factor- β (4, 5). The glycoprotein hormone α -subunit is encoded in a single gene and thus identical in the amino acid sequence within a species. In contrast, the β -subunits are unique, encoded in distinct genes and responsible for biological specificity (6, 7).

Assembly of the α - and β -subunits is an early posttranslational event in glycoprotein hormone synthesis occurring in the endoplasmic reticulum (8). Heterodimerization is critical for disulfide bond formation and for hormone-specific posttranslational modifications, such as processing of the carbohydrate side chains, and thus rate-limiting for the secretion of glycoprotein hormones (9, 10). Moreover, dimer formation is essential for hormonal activity, since free subunits have minimal receptor binding affinity (1). In addition, dissociation of heterodimeric glycoproteins into their subunits may be a significant factor in terminating glycoprotein hormone activity *in vivo* (11).

Therefore, covalent linking of the glycoprotein hormone subunits should overcome assembly-dependent deficiency in secretion and may increase hormone stability and activity. It has recently been pioneered by Boime and colleagues and subsequently shown by the group of Puett that bioactive gonadotropins could be produced as single chains (12–14), but it is not clear whether this approach is applicable for hTSH, or whether such fusion would affect the stability or the *in vivo* clearance of these hormones. Such fusion should be particularly relevant to TSH, since the free TSH β -subunit, in contrast to the free CG β -subunit, is unstable in the monomeric form and degraded intracellularly unless stabilized by dimerization with the α -subunit (15). Here, we show that it is possible by subunit gene fusion to produce a tethered form of hTSH with comparable *in vitro* activity to dimeric hTSH. Furthermore, fusion significantly increased the stability and prolonged the *in vivo* half-life of hTSH. Moreover, the expression of hTSH as a single chain could overcome selected mutagenesis-induced defects in hTSH secretion, and thus, this approach may be used to expand the spectrum of structure-function studies of glycoprotein hormone analogs. Subunit gene fusion therefore appears to be a promising strategy, not only for the generation of long lasting

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¹ The abbreviations used are: TSH, thyroid-stimulating hormone; hTSH, human thyroid-stimulating hormone; hTSH β , human thyroid-stimulating hormone β subunit; CG, chorionic gonadotropin; FSH, follicle-stimulating hormone; rh, recombinant human; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; wt, wild type; CTP, carboxyl-terminal peptide; SC, single chain; CMV, cytomegalovirus.

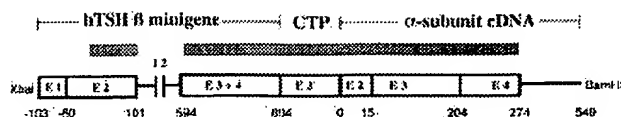


Fig. 1. **hTSH-SC construct.** The hTSH β minigene bearing the 32-amino acid CTP of the hCG β -subunit was fused to the α -subunit cDNA by overlap extension PCR as described under "Experimental Procedures." E, exon; I, intron. The numbers below denote the base pairs corresponding to the respective subunit genes and the gray bar above represents the coding region of the mature protein.

hTSH analogs, but particularly for the development of recombinant mutants with desirable characteristics, the utility of which may be limited by decreased stability.

EXPERIMENTAL PROCEDURES

Materials.—CHO cells stably transfected with the hTSH receptor (clone JP09) were kindly donated by Dr. G. Vassart, Belgium, and FRTL-5 cells expressing the endogenous rat TSH receptor by Dr. L. D. Kohn, Interthyr Research Foundation (Baltimore, MD). cAMP antibody was generously supplied by Dr. J. L. Vaitukaitis, National Institutes of Health (Bethesda, MD). Cell culture media and reagents were purchased from Life Technologies, Inc., and [125 I]-cAMP and [125 I]-hTSH radiolabeled to a specific activity of 40–60 μ Ci/ μ g from Hazleton (Vienna, VA). PCR reagents were obtained from Boehringer Mannheim and New England Biolabs (Beverly, MA).

Site-directed Mutagenesis.—The construction of the hTSH β -subunit bearing the carboxyl-terminal extension peptide of the hCG β -subunit (hTSH β -CTP) has been described previously (16). To produce single chain hTSH (hTSH-SC), we used overlap extension PCR (17) to fuse the amino terminus of the α -subunit cDNA (without the signal sequence) to the carboxyl-terminal end of the hTSH β -CTP (Fig. 1). Primers P2 5'-CAC ATC AGG AGC TTG TGG GAG GAT CGG and P3 5'-ATC CTC CCA CAA GCT CCT GAT GTG CAG span both the carboxyl-terminal end of the hTSH β -subunit containing the hCG β -CTP (hTSH β -CTP) as well as the amino terminus of the coding sequence of the α -subunit. In addition, P1 5'-CTC GAG TCT AGA ATG ACT GCT CTC TTT CTG ATG was designed to anneal 5' of the hTSH-CTP minigene signal peptide, and P4 5'-CGA CGT GGA TCC ATG CTG TAT TCA TTC to anneal 3' of the hTSH β -subunit cDNA. Initially, two PCR reactions were performed: P1 and P2 were used with the hTSH-CTP as the template (PCR no. 1), and P3 and P4 using the α -subunit cDNA (PCR no. 2). In a third PCR reaction (PCR no. 3), both these overlapping products were used as combined template to generate the single chain hTSH-SC with P1 and P4.

To create Gln⁶²-Gln⁷⁸/TSH β -SC in which both α -glycosylation recognition sequences were deleted by mutating both Asn⁵⁵² and Asn⁶⁷⁸ to Gln, a previously described α -subunit cDNA construct (Gln⁵⁵²-Gln⁷⁸) (18) was used as the template for PCR no. 2. Similarly, to obtain Asp³³⁹/TSH β -SC, the α -subunit cDNA construct Asp⁶³⁸ (19) served as the template in PCR no. 2. Following subcloning of the fused wild type or mutant 2-kilobase pair hTSH-SC constructs into the pLB-CMV expression vector, the entire PCR product was sequenced in each case to rule out any undesired polymerase errors.

Transient Expression.—CHO-K1 Cells (ATCC, Rockville, MD) were maintained in Ham's F-12 medium supplemented with 5% fetal calf serum, penicillin (50 units/ml), streptomycin (50 μ g/ml) and glutamine (4 mM). To obtain dimeric wild-type hTSH (hTSH-wt), cells were cotransfected in 60-mm culture dishes with the α -subunit cDNA in pcDNA 1/neo and the hTSH β minigene in the pLB-CMV vector, using a total amount of 2 μ g DNA per dish and a liposome formulation (LipofectAMINE reagent, Life Technologies, Inc.) as described previously (20). The hTSH-SC fusion products in the pLB-CMV vector were transfected with identical amounts of total DNA. On the following day, the transfected cells were transferred to CHO serum-free medium (Life Technologies, Inc.). After an additional 48 h, the supernatants, including control medium from mock transfections using the expression plasmids without gene inserts, were harvested. The collected media were then concentrated using a Centrprep 10 concentrator (Amicon, Beverly, MA) and used for immunoassays, the various activity assays, and clearance studies.

Immunoassays of hTSH.—The hTSH constructs were quantified with a panel of different immunoassays, using a total of four different hTSH immunoassays utilizing different monoclonal antibodies, which were described in detail previously (19, 21).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting.—Conditioned media from transiently transfected CHO cells were con-

centrated, fractionated on ConA-Sepharose columns (Pharmacia), re-concentrated, and denatured by boiling in 0.25% SDS, 0.5% β -mercaptoethanol. Samples were then resolved on 14% Tris-glycine polyacrylamide gels, transferred to nitrocellulose membranes, and incubated overnight with a polyclonal rabbit antibody directed against the hTSH α -subunit (18). Antigen-antibody complexes were subsequently visualized by chemiluminescence using a horseradish peroxidase-coupled anti-rabbit IgG and a luminol substrate (Boehringer Mannheim).

Radioreceptor Assay of hTSH.—The receptor-binding activity of the various hTSH constructs was determined by their ability to displace [125 I]-hTSH from solubilized porcine thyroid membrane receptor preparations (Kronus, Dana Point, CA) following the manufacturer's instructions. Binding was also studied in whole cells using FRTL-5 cells expressing the endogenous rat TSH receptor, as described previously (20).

cAMP Production in JP09 Cells.—CHO cells stably expressing the rhTSH receptor (JP09) were grown in 96-well culture plates in Ham's F-12 medium supplemented as above. Confluent cells were incubated for 2 h at 37 °C, 5% CO₂, with serial dilutions of hTSH constructs or control medium from mock transfections in a modified Krebs-Ringer buffer supplemented with 280 mM sucrose to maintain isotonicity and 1 mM 3-isobutyl-1-methylxanthine. The amount of cAMP released into the medium was determined by radioimmunoassay (20).

cAMP Production in FRTL-5 Cells.—FRTL-5 cells were maintained as described elsewhere (20) and, prior to the cAMP assay, grown in 96-well culture plates in the absence of TSH for 6–8 days. cAMP production of hTSH constructs was determined using the protocol for JP09 cells.

Growth Assay in FRTL-5 Cells.—FRTL-5 cells were grown in 24-well plates in the presence of TSH to 30% confluence and then cultured in TSH-free medium for 4 days. Subsequently, the cells were incubated with serial dilutions of hTSH constructs or control from mock transfected cells. After 48 h, 1.0 μ Ci of [3 H]thymidine per well (DuPont) was added, and the cells were incubated for an additional 24 h. Subsequently, [3 H]thymidine uptake measured as described previously (18).

Plasma Clearance Rate.—The clearance rate of the hTSH constructs was determined in the rat after intravenous injection of the different hTSH preparations and subsequent determination of hTSH serum levels at defined intervals from 1 to 120 min. Experimental details of this procedure have been described previously (22, 23).

RESULTS

Genetic Fusion of the hTSH α - and β -Subunit.—Truncation as well as amino acid mutation studies had previously indicated the importance of the α -carboxyl terminus for hTSH activity (20). To maintain accessibility of this region, we fused the carboxyl terminus of the TSH β -subunit to the amino terminus of the α -subunit. We also included the CTP of the hCG β -subunit, here defined as the carboxyl-terminal 32 amino acids of the hCG β -subunit. The CTP has a high proline/serine content, which lacks significant secondary structure and was previously shown to be suitable as a flexible linker for efficient expression of single chain hFSH (13). In keeping with previous observations, addition of CTP to the hTSH β -subunit was predicted not to affect receptor binding or intrinsic activity of hTSH (16). Since addition of the CTP had previously been shown to prolong the half-life of hTSH, the clearance rate of hTSH-SC was compared with both dimeric hTSH-wt as well as hTSH-CTP (see below).

Effect of Subunit Fusion on hTSH Secretion.—To demonstrate that hTSH-SC was indeed produced and secreted as a single chain, we performed SDS-polyacrylamide gel electrophoresis and subsequent Western blotting of ConA-fractionated conditioned media from CHO cells transiently transfected with either the fusion product or individual hTSH subunits using an antibody against the α -subunit. Under reducing conditions, heterodimeric hTSH-wt dissociated into individual subunits, and the free α -subunit migrated at the expected 25 kDa. In contrast, the α -subunit antibody recognized a 55-kDa band consistent with the covalently linked hTSH fusion protein (Fig. 2). The level of secretion of hTSH-SC from transiently transfected CHO cells, as determined by four different immunoassays, was similar to hTSH-wt (Table I), if individual subunit plasmids were cotransfected at a 3 to 1 molar ratio. Such

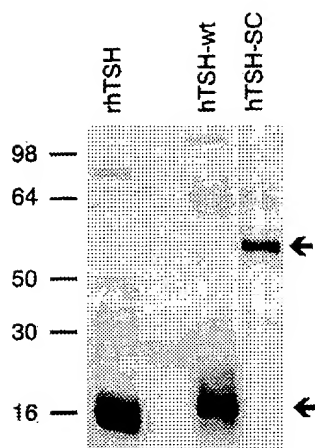


FIG. 2. Western blot analysis of ConA-Sepharose-fractionated hTSH-wt and hTSH-SC obtained from conditioned media harvested from transiently transfected CHO cells. Also shown, as an internal standard, is rhTSH, kindly provided by the Genzyme Corp. (Cambridge, MA). A polyclonal rabbit antibody against the hTSH α -subunit was used. Under the reducing conditions used, dimeric hTSH dissociates into individual subunits, and the free α -subunit migrated as the expected 25-kDa protein (bottom arrow). In contrast, the hTSH-SC migrated at 55 kDa, consistent with the size of a linked α - β subunit complex (top arrow). The presence of nonprominent higher molecular weight bands was consistently observed with the different hTSH preparations as well as mock transfected supernatant from independent transient transfections and therefore most likely due to nonspecific antibody interaction. Therefore, a potential specific effect on the biological or physical properties of a hTSH preparation would not be expected.

TABLE I
Secretion of hTSH analogs

Hormone levels were determined in conditioned media from CHO cells transiently transfected with the respective hTSH constructs. *n* denotes number of independent transfections, each performed in triplicate dishes. Free α -subunit levels were similar in conditioned media from hTSH-wt and Asp³³⁸/hTSH β -wt, but not detectable in media from either Gln⁵⁵²-Gln⁷⁸/hTSH β -wt or Gln⁵⁵²-Gln⁷⁸/hTSH β -SC (see text).

Analog	Subunit plasmid ratio	Secretion ng/ml \pm S.E.	n
hTSH-wt	α : β 3:1	31.2 \pm 1.7	5
	α : β 1:1	24.1 \pm 1.6	3
hTSH-CTP	α : β 3:1	25.2 \pm 2.8	5
	α : β 1:1	17.1 \pm 1.1	3
hTSH-SC		29.6 \pm 4.7	11
Gln ⁵⁵² -Gln ⁷⁸ /hTSH β -wt		0.05 \pm 0.05	4
Gln ⁵⁵² -Gln ⁷⁸ /hTSH β -SC		5.1 \pm 1.8	5
Asp ³³⁸ /hTSH β -wt		<0.01	3
Asp ³³⁸ /hTSH β -SC		<0.01	3

a 3 to 1 molar excess of the α -subunit plasmid led to a higher secretion of dimeric hTSH compared with transfection of both subunits at an equimolar ratio. Addition of the CTP to the hTSH β -subunit reduced secretion of dimeric hTSH, whereas fusion of the hTSH subunits with inclusion of the CTP sequence as a linker did not impair subunit folding or expression of the hormone (Table I).

Effect of Subunit Fusion on Secretion-deficient hTSH α -Subunit Mutants—To test the effects of subunit fusion on mutagenesis-induced defects in hTSH secretion, we studied the secretion of single chain hTSH analogs Gln⁵⁵²-Gln⁷⁸/hTSH β -SC lacking the two α -subunit glycosylation recognition sequences and Asp³³⁸/hTSH β -SC. These mutations had previously been shown to profoundly decrease or abolish the secretion of dimeric hTSH (18, 19) (Table I). Consistent with findings that non-glycosylated glycoprotein hormone subunits are misfolded and degraded intracellularly, the free Gln⁵⁵²-Gln⁷⁸ subunit

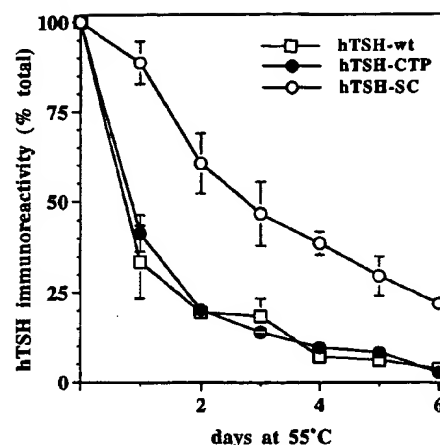


FIG. 3. hTSH stability. hTSH immunoreactivity, measured as percent of total remaining hTSH, was determined for hTSH-wt, hTSH-CTP, and hTSH-SC at 55 °C for 6 days using an assay specific for dimeric hTSH without cross-reactivity to free subunits. Values are the mean \pm S.E. of three independent experiments, each performed in duplicate. At 37 °C, all hTSH constructs were stable (<5% degradation) for at least 21 days. In some cases, no error bar is visible because it is equivalent to or smaller than the size of the respective symbol.

was not detectable (<0.01% of hTSH-wt-free α -subunit) by an α -subunit-specific radioimmunoassay. In contrast, free Asp³³⁸ subunit was secreted in levels quantitatively similar to hTSH-wt free α -subunit (19), suggesting that the failure of the Asp³³⁸ subunit to dimerize with hTSH β -subunit was not related to its misfolding or degradation. Fusion of the Gln⁵⁵²-Gln⁷⁸ subunit to the hTSH β -subunit increased secretion, indicating that fusion of both subunits can partially overcome the requirement of α -subunit carbohydrate chains for hTSH secretion. In contrast, fusion of the Asp³³⁸ subunit did not increase the amount of hTSH produced, suggesting that this particular mutation, possibly due to its predicted location at the subunit interface in close proximity to residues forming intersubunit hydrogen bonds (2, 3) prevents subunit association (Table I).

Effect of Subunit Fusion on hTSH Stability—Stability of the different hTSH proteins was tested initially by incubating conditioned media obtained from transient transfections at 37 °C. All three forms of hTSH, hTSH-wt, hTSH-CTP as well as hTSH-SC were stable at this temperature, and there was minimal (<5%) degradation over a period of 21 days, as judged by repeated determinations of hTSH immunoreactivity with an assay specific for heterodimeric hTSH, which does not recognize free subunits. However, incubation at 55 °C showed that the fused hTSH-SC was significantly more stable than dimeric hTSH in that less than 15% of hTSH-SC was degraded after 24 h, compared with more than 50% of dimeric hTSH, either hTSH-wt or hTSH-CTP (Fig. 3).

Effect of Subunit Fusion on Receptor Binding and Intrinsic Activity of hTSH—The receptor binding of the fused hTSH-SC was similar to that of hTSH-wt and hTSH-CTP when tested in porcine thyroid membranes (Fig. 4) or in FRTL-5 cells expressing the endogenous rat TSH receptor (not shown). In addition, the ability of hTSH-SC to induce cAMP stimulation in JP09 cells (Fig. 5a), as well as cAMP stimulation (Fig. 5b) and growth promotion (Fig. 5c) in FRTL-5 cells was comparable to that of hTSH-wt and to that of hTSH-CTP. This indicates that both introduction of the CTP linker as well as subunit fusion did not alter the *in vitro* characteristics of hTSH.

Effect of Subunit Fusion on hTSH Clearance—In accord with previous studies from our laboratory (16), addition of the CTP to the hTSH β -subunit significantly prolonged the plasma half-life of dimeric hTSH. 50% of the hTSH-CTP was cleared from

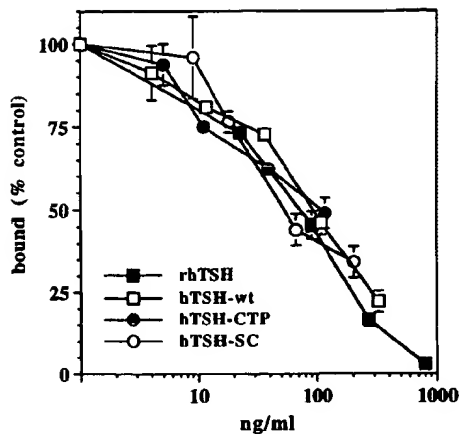


FIG. 4. Inhibition of ^{125}I -bTSH receptor binding by the hTSH preparations. Increasing doses of hTSH were incubated with porcine membranes in the presence of a constant amount of ^{125}I -bTSH. ^{125}I -bTSH bound to membranes was precipitated and quantitated in a γ counter. Radioactivity precipitated in the presence of concentrated medium from mock transfections was defined as 100%. rhTSH was obtained from the Genzyme Corp. Values are the mean \pm S.E. of three independent experiments, each performed in at least duplicate. Also see legend to Fig. 3.

the rat circulation after 23.2 ± 7.9 min compared with 8.7 ± 6.1 min for hTSH-wt ($p = 0.01$). Remarkably, fusion of the individual subunits including the CTP as a linker led to an even further significant prolongation of half-life; 50% of hTSH-SC was cleared after 51.6 ± 14.4 min ($p = 0.02$ compared with hTSH-CTP) (Fig. 6).

DISCUSSION

The half-life of recombinant analogs can be prolonged by increasing the Stoke's radius of a protein using polyethylene glycolylation or the introduction of new carbohydrate recognition sites, by modification of protease recognition sites to increase stability, or by carbohydrate modification to avoid carbohydrate-specific clearance mechanisms (5, 21, 23). Our present study using a genetically fused, single chain hTSH highlights a novel way by which an increased *in vivo* half-life may be achieved.

Although it had previously been shown that bioactive hCG and hFSH could be produced as a single chain (12–14), the effect of genetic fusion on glycoprotein hormone stability and plasma clearance rate had not previously been investigated. Further, from the findings on hCG and hFSH, it was not predictable whether a fusion approach would also be feasible for hTSH. In particular, recent mutational analysis of hTSH structure-function relationships has identified common α -subunit domains that play strikingly different roles for heterodimer formation, receptor binding, and bioactivity of hTSH compared with hCG and hFSH (18–20). Interestingly, these domains are located in close proximity to the β -seat-belt region, which is crucial for hTSH specificity, suggesting that the seat-belt may direct these common domains to function in a hormone-specific fashion (5, 24).

In light of previous observations (16), validated here, that addition of the CTP with its *O*-linked carbohydrate side chains prolonged hTSH half-life *in vivo*, the full-length CTP was used as a linker for fusing the hTSH subunits. We anticipated that the linker may synergize with the fusion to prolong the half-life of hTSH *in vivo*. Indeed, gene fusion significantly decreased the clearance rate of dimeric hTSH even when compared with dimeric hTSH bearing the CTP. This indicates that dissociation of hTSH into its subunits occurs *in vivo* and contributes to its deactivation, as individual subunits are devoid of *in vivo* activ-

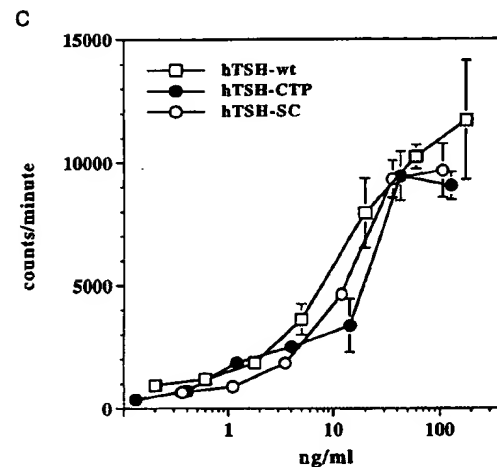
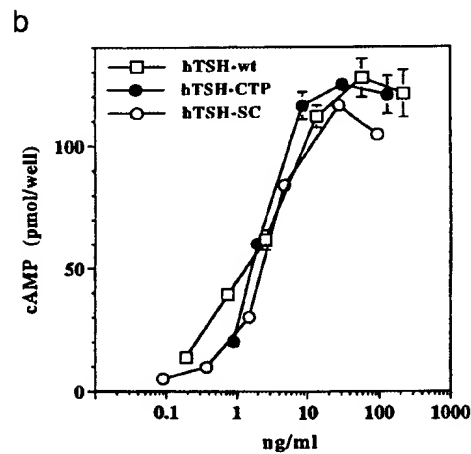
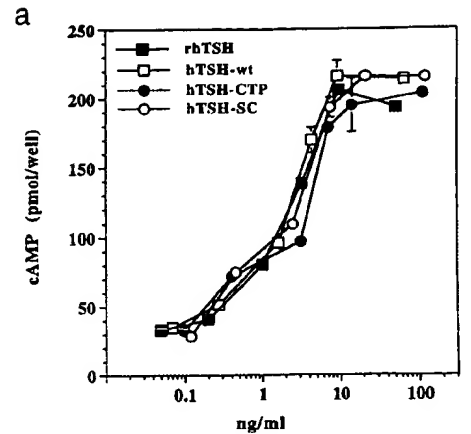


FIG. 5. *a* and *b*, cAMP induction by the various hTSH constructs in JP09 cells (*a*) and FRTL-5 cells (*b*). Increasing concentrations of the various hTSH constructs were incubated with JP09 or FRTL-5 cells, and the cAMP concentration in the resulting supernatants was assayed by radioimmunoassay. *c*, induction of cell growth by the hTSH constructs. Increasing concentrations of hTSH were incubated with FRTL-5 cells, which were previously grown in the absence of TSH. After 48 h, ^3H thymidine was added, and after an additional 24 h, radioactivity incorporated into the DNA was measured. The radioactivity incorporated by the cells in the presence of concentrated medium from mock transfected cells was not different from base line levels. rhTSH represents recombinant hTSH from Genzyme Corp. Values are shown as the mean of triplicate observations \pm S.E. See also the legend to Fig. 3.

ity and rapidly cleared from the circulation (11).

In addition, fusion of the subunits of hTSH increased its thermostability. It is conceivable that such enhanced stability

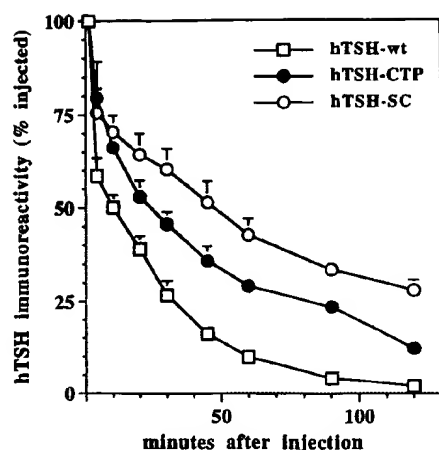


FIG. 6. Serum disappearance rate of the various hTSH constructs in male rats. After bolus injection of 200–300 ng of hTSH into the femoral vein, blood for hTSH determinations was obtained over 120 min at equal time points. An immunoradiometric assay without cross-reactivity to rat TSH (Nichols Institute), was used. Immunoreactivity was expressed as mean \pm S.E. percent remaining, and serum concentration at 0 min was defined as 100%. A total of $n = 5$ animals was used for each hTSH preparations. See also the legend to Fig. 3.

may become particularly relevant for recombinant glycoprotein hormone analogs with genetically engineered novel features that are less stable than the wild-type hormone. In this respect, hFSH analogs have recently been described, in which site-directed mutagenesis within regions important for activity significantly decreased their stability (25).

Moreover, genetic subunit fusion can overcome certain mutagenesis-induced defects in heterodimer formation. The presence of carbohydrate side chains on both subunits is essential for proper subunit folding and combination, and intracellular assembly of deglycosylated subunits is inefficient (10). Indeed, glycosylation of the α -subunit appears necessary to overcome retention of the hTSH β -subunit in the endoplasmic reticulum (8), and in contrast to the free hCG β -subunit, the free hTSH β -subunit is not efficiently secreted (26). Our fusion experiments suggest that the glycosylated hTSH β -subunit, if fused to an α -subunit devoid of glycosylation recognition sequences, may function as a chaperone inducing α -subunit folding despite the absence of carbohydrate chains and thus partially rescue the nonglycosylated α -subunit. On the other hand, fusion was not able to induce heterodimer formation with a mutated α -subunit Asp³⁸ which, although dimer formation-incompetent, nevertheless appeared to be properly folded and secreted.

It is interesting to consider the dimeric structure of glycoprotein hormones from an evolutionary perspective. The glycoprotein hormones were probably derived from a common ancestor gene, and in less developed organisms, a single primordial monomeric hormone with a corresponding receptor was likely sufficient for the necessary endocrine functions (27). To fulfill the requirements for an increasingly complex organism, adopting a dimeric ligand structure enabled functional diversification and increased flexibility without the need for the development of entirely new mechanisms of receptor activation, albeit perhaps at the expense of reduced protein stability. This diversification appears to have evolved by the emergence of inhibitory domains on both ligand and receptor which impose steric hindrances thus allowing only the intended li-

gand to interact with the common activation domain (28). Such negative specificity determinants have not only developed in glycoprotein hormones and their receptors, but also in other members of the cystine knot growth factor superfamily, such as neurotrophins (29), and also in other G protein-coupled receptors (30). More generally, dimer formation is necessary for the activity and specificity of many, if not all cystine knot growth factors, as well as for other bioactive molecules, such as enzymes and transcription factors. In this respect, fusion of individual protein monomers has recently been used to develop transcription factors and cytokine analogs with defined properties and increased biological activities (31, 32). This approach poses a universal strategy to enhance both stability and bioactivity as well as to control specificity of noncovalently linked oligomers, and may also be used to engineer molecules with novel activities or specificities.

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